

A Genetic Screen for Synaptic Transmission Mutants Mapping to the Right Arm of Chromosome 3 in *Drosophila*

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ABSTRACT

Neuronal function depends upon the proper formation of synaptic connections and rapid communication at these sites, primarily through the regulated exocytosis of chemical neurotransmitters. Recent biochemical and genomic studies have identified a large number of candidate molecules that may function in these processes. To complement these studies, we are pursuing a genetic approach to identify genes affecting synaptic transmission in the *Drosophila* visual system. Our screening approach involves a recently described genetic method allowing efficient production of mosaic flies whose eyes are entirely homozygous for a mutagenized chromosome arm. From a screen of 42,500 mutagenized flies, 32 mutations on chromosome 3R that confer synaptic transmission defects in the visual system were recovered. These mutations represent 14 complementation groups, of which at least 9 also appear to perform functional roles outside of the eye. Three of these complementation groups disrupt photoreceptor axonal projection, whereas the remaining complementation groups confer presynaptic defects in synaptic transmission without detectably altering photoreceptor structure. Mapping and complementation testing with candidate mutations revealed new alleles of the neuronal fate determinant *svp* and the synaptic vesicle trafficking component *lap* among the collection of mutants recovered in this screen. Given the tools available for investigation of synaptic function in *Drosophila*, these mutants represent a valuable resource for future analysis of synapse development and function.

MANY of the factors responsible for axonal pathfinding, synapse formation, and synaptic function in metazoans were first identified in classical genetic screens carried out in *Drosophila*. While the genetic screening approaches used to identify these factors are powerful, they have several significant limitations. Most notably, screens for axonal pathfinding components are highly labor intensive, requiring the generation of mutagenized lines and the systematic screening of individual lines using antibody- or green fluorescent protein (GFP)-based methods to identify those with altered neuronal structure (SEEGER *et al.* 1993; ZALLEN *et al.* 1999; PARNAS *et al.* 2001). Although somewhat less labor intensive, classical genetic screens for *Drosophila* mutants with altered neuronal function have primarily resulted in the identification of genes that function only in the

visual system (HOTTA and BENZER 1969; PAK *et al.* 1969; HEISENBERG 1971) or have been carried out under conditions favoring the recovery of conditional alleles (SUZUKI 1970; SIDDIQI and BENZER 1976), and thus only a fraction of the genes involved in synaptic transmission were recovered from these studies. More powerful genetic screening approaches in *Caenorhabditis elegans* that circumvent some of the limitations of the *Drosophila* system have also been successfully used to identify factors involved in synaptic development and function (BRENNER 1974; JORGENSEN and MANGO 2002). However, the subsequent analysis of some of the mutants recovered from these screens has been compromised somewhat by the difficulty in conducting electrophysiological analysis in *C. elegans*.

Over the past decade, targeted mutagenesis of candidate genes has largely supplanted classical genetic analysis of neurotransmitter release mechanisms owing to rapid progress in the biochemical identification of components thought to act in this process (FERRO-NOVICK and JAHN 1994; FERNANDEZ-CHACON and SUDHOF 1999; LIN and SCHELLER 2000). The recent completion of the *C. elegans*, *Drosophila*, and mouse genome projects has further added to the list of genes that may function in neurotransmitter release (LLOYD *et al.* 2000). These approaches have led to the identification and characterization of components that act at many stages of synaptic

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vesicle trafficking, including vesicle fusion with the presynaptic membrane in response to calcium influx and vesicle recycling following fusion (FERNANDEZ-CHACON and SUDHOF 1999; LLOYD *et al.* 2000; RICHMOND and BROADIE 2002). While these studies have provided insight into the mechanisms of neurotransmitter release, a potential limitation of this approach is that it is likely biased in favor of those factors most readily amenable to biochemical analysis. Another challenge with this approach is that it often takes substantial time and effort to generate mutations in the genes of interest and, in several cases, the resulting mutants have little or no phenotype (ROSAHL *et al.* 1993; GEPPERT *et al.* 1994; McMAHON *et al.* 1996) or display phenotypes unrelated to presynaptic function (LEVENTIS *et al.* 2001; RAZZAQ *et al.* 2001; ZELHOF *et al.* 2001; ANDREWS *et al.* 2002; MURTHY *et al.* 2003).

Recently, some of the limitations of the previous classical genetic and biochemical approaches have been overcome by the development of the *EGUF/hid* system (STOWERS and SCHWARZ 1999). This system combines the *FLP/FRT* method of generating mosaic tissues in *Drosophila* with the *GAL4/UAS* system to target mitotic recombination to cells that make up the retina of the compound eye. The presence of an eye-specific, dominant, proapoptotic factor eliminates all cells that do not bear homozygous clones of a mutated chromosome arm. This system can be used to generate F₁ progeny from a mutagenized parent that are homozygous for a mutagenized chromosome arm in the retina but heterozygous elsewhere. Subsequent screening to identify flies with phototactic defects and electroretinogram alterations indicative of a defect in synaptic transmission can be used to recover mutants of interest.

In this study, we describe the preliminary results of a screen for mutations mapping to the right arm of chromosome 3, representing approximately one-fifth of the *Drosophila* genome, that result in presynaptic defects in synaptic transmission. From this screen, 14 complementation groups were identified, of which 11 appear to specifically affect presynaptic function and 3 affect axonal pathfinding. All of these mutations have been mapped and nearby candidate genes have been identified and, where possible, tested for complementation with our mutants. One of the complementation groups exhibiting an aberrant axonal projection pattern appears to represent the *seven up* gene, and one with unaltered photoreceptor structure represents the *lap* gene. This work provides a foundation for the identification of novel components involved in neuronal development and function.

MATERIALS AND METHODS

***Drosophila* husbandry:** All crosses were carried out at 22°, and stocks were maintained on cornmeal agar. The stocks used in

this analysis were obtained from the Bloomington *Drosophila* Stock Center or Berkeley *Drosophila* Genome Project.

Generation of mutants: One- to three-day-old male flies carrying an *FRT* element inserted at polytene segment 82B were mutagenized by feeding an EMS-containing sucrose solution as described (GRIGLIATTI 1998). Mutagenized males were then crossed to virgin females of one of the following genotypes: *y, w; EGUF/EGUF; FRT82B, GMR-hid/TM6C* or *y, w; EGUF/EGUF; FRT 82B, GMR-hid/TM6,y+* (both genotypes hereafter collectively designated *EGUF-hid 3R*). Male *nonbalancer* chromosome offspring from these crosses were subjected to an assay of phototaxis (described below).

Mutants with *sd¹-sd¹⁵* allele designations were recovered by placing ~100 mutagenized flies with normal eye morphology into a countercurrent apparatus (BENZER 1967) and providing flies 15 sec to move at least half the distance of the apparatus toward a light source. This phototactic selection was repeated five times. Flies that failed to move toward the light in at least three trials were recovered and tested again in identical fashion the following day. Flies exhibiting phototactic defects on successive days were individually mated to the *EGUF-hid 3R* stock to generate a population of flies bearing the same mutagenized chromosome and assayed for phototactic defects as described above. Mutants exhibiting phototactic defects as a population were crossed to a *TM6B/TM3* stock to recover the mutagenized chromosome over the *TM6B* balancer chromosome. From a screen of 18,500 mutagenized flies, 171 phototactic mutants were recovered of which 15 appear to be specifically defective in synaptic transmission on the basis of the results of electroretinogram recordings (see below).

Mutants with *sd¹⁶-sd³²* allele designations were isolated by placing up to 500 mutagenized flies into a 500-ml flask and providing flies 15–20 sec to move into an adjacent 500-ml flask toward a fluorescent light source. Flies with normal external eye morphology that failed phototactic selection on two successive trials were retested in identical fashion the following day. Flies that again failed the phototaxis selection were individually mated to *EGUF-hid 3R* females. Appropriate progeny from this cross were subjected to electroretinogram recordings to identify mutants with defects in synaptic transmission as described below. Mutants exhibiting the desired electroretinogram characteristics were mated to *y w; Sp/CyO y+; Ly/TM6 y+* females to recover chromosomes of interest *in trans* to the *TM6 y+* balancer chromosome. From a screen of 24,000 mutagenized flies, 17 mutants with presynaptic defects in synaptic transmission were recovered.

Electroretinogram analysis of mutants: Balanced stocks bearing the mutations conferring nonphototactic phenotypes were crossed to the *EGUF-hid 3R* stock to generate offspring possessing eyes homozygous for the relevant chromosome. Electroretinogram recordings were carried out on these flies following dark adaptation as described (PAK *et al.* 1969).

Complementation analysis, mapping, and lethal phase analysis of synaptic transmission mutants: Complementation analysis was carried out in two ways: First, balanced stocks of each of the different mutants were crossed to one another in all possible combinations and offspring were scored for the presence of viable nonbalancer progeny. Mutations that failed to complement each other regarding viability were considered allelic. For those mutants that produced viable offspring from the complementation crosses, *trans*-heterozygous nonbalancer offspring were subjected to an assay of phototaxis as described above. Mutations that failed to complement each other for the phototaxis phenotype were further characterized by conducting electroretinogram (ERG) recordings to confirm their allelic relationship.

Deficiency mapping was conducted by crossing balanced stocks of all of the synaptic transmission mutants to a collection

of deficiency stocks, which together span most of the right arm of chromosome 3. Mutations were tentatively assigned to the deficiency intervals of those deficiencies that failed to produce viable hemizygous offspring. Recombinational mapping was carried out by crossing the synaptic transmission mutants to a stock bearing an *FRT* element at polytene position 82B and the recessive markers *ru¹*, *h¹*, *th¹*, *st¹*, *cu¹*, *sr¹*, *e*, and *ca¹*. Female offspring from this cross were then mated to a stock lacking the *FRT* element at 82B but bearing the same recessive markers to identify recombinants. A total of 100 male recombinants from this cross were then selected and individually mated to the *EGUF-hid* 3R stock. The appropriate male offspring from these crosses were subjected to a test of phototaxis as described above. The map positions of mutations responsible for phototactic phenotypes were calculated by determining the average recombination frequency and standard deviations obtained from linked markers. Mutations that produced consistent results in the deficiency and recombinational mapping exercise were assigned a localization defined by the deficiencies that fail to rescue the recessive lethal phenotype and in some cases were further delimited by overlapping complementing deficiencies.

Those mutations that produced conflicting results in the deficiency and recombinational mapping exercise or that complemented all of the deficiency chromosomes were crossed again to deficiencies mapping to the polytene regions implicated from recombinational mapping. Viable offspring from these crosses were tested for phototactic and ERG phenotypes as described above. Those mutations that complemented all of the deficiencies tested were subjected to further recombinational mapping to refine the genetic map position. Recombinational mapping was performed as described above, but only animals with recombinational events near linked markers were used in this analysis. Mutations were localized to the cytological intervals defined by deficiency mapping experiments or were assigned a genetic map position determined from the average recombination frequency and standard deviations obtained from linked markers if they complemented all of the available deficiencies.

To facilitate lethal-phase analysis, mutant chromosomes were placed *in trans* to a balancer chromosome marked with GFP. Homozygous non-GFP offspring from each stock were collected and monitored until lethality occurred. In addition, those mutants that map to deficiencies were crossed to stocks bearing the relevant deficiency chromosome *in trans* to a GFP-marked balancer chromosome. In both analyses, non-GFP offspring were monitored until lethality occurred.

Candidate genes corresponding to the mutations recovered in this analysis were tested by obtaining stocks bearing mutations in the candidate genes (where possible) and by performing complementation analysis.

Analysis of photoreceptor axonal projection patterns: Heads were prepared for immunohistochemistry by removing the proboscis and air sacs ventral to the brain and then fixing in PBS plus 4% paraformaldehyde for 3 hr at 4°. Fixed heads were then rinsed 10 min in PBS plus 12% sucrose, incubated in 25% sucrose overnight at 4°, and then frozen in OCT freezing medium (VWR) and sectioned at 10–12 μ m. Head sections were rinsed two times for 5 min each in PBST (PBS plus 0.5% Triton X-100), blocked 1 hr at room temperature in PBSTNGS (PBST plus 5% normal goat serum), and then incubated 2 hr at room temperature (or overnight at 4°) with a 1:50 dilution of mAb24B10 (obtained from Developmental Studies Hybridoma Bank, University of Iowa; FUJITA *et al.* 1982). Following incubation with the primary antiserum, head sections were washed three times for 5 min each in PBST and incubated with a 1:300 dilution of a goat anti-mouse

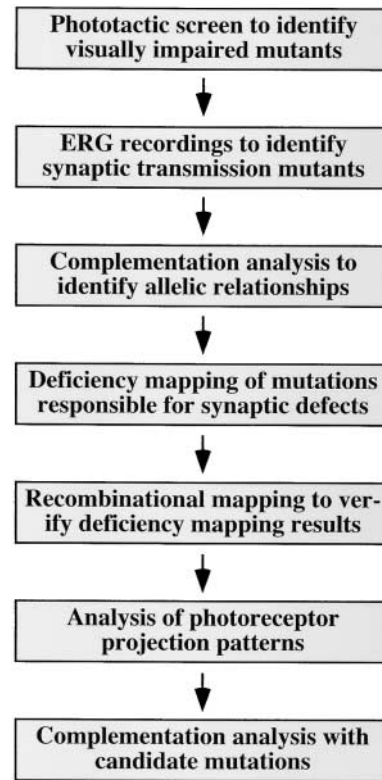


FIGURE 1.—Flow chart diagramming the identification and characterization of photoreceptor synaptic transmission defective mutants recovered in this work.

horseradish peroxidase-coupled secondary antibody (Jackson ImmunoResearch, Bar Harbor, ME) in PBSTNGS for 2 hr at room temperature. Following incubation with the secondary antiserum, head sections were washed three times for 5 min in PBST and developed for 10 min in PBS containing 0.5% diaminobenzidine and 0.03% hydrogen peroxide. Head sections immunostained for synaptotagmin were processed as above, except that a rabbit anti-synaptotagmin antiserum was used at 1:1000 as the primary antiserum (a gift from Troy Littleton; LITTLETON *et al.* 1993) with a horseradish peroxidase-coupled goat anti-rabbit secondary antibody (Jackson ImmunoResearch).

RESULTS

Screening for synaptic transmission mutants: To identify mutants with defective synaptic function in the visual system, male flies homozygous for an *FRT* element at polytene position 82B were mutagenized with EMS and crossed to *EGUF-hid* 3R females (see Figure 1 for an overview of the screen and MATERIALS AND METHODS for further details). A total of 42,500 F₁ offspring from this cross, homozygous for the right arm of chromosome 3 in the retina, were subjected to a test of phototaxis. Nonphototactic flies with normal external eye morphology were recovered and crossed to the *EGUF-hid* 82B stock to produce a population of offspring bearing the same mutagenized chromosome. These F₂ flies were

then tested as a population to verify the original phototactic phenotype (*sd*¹⁻¹⁵ alleles) or directly subjected to electrophysiological analysis (*sd*¹⁶⁻³² alleles).

Mutants with defects in synaptic transmission were identified from among the collection of phototactic mutants by carrying out ERG recordings. The ERG monitors electrical activity in the compound eye in response to light and consists of the sum of two components: a negative component corresponding to phototransduction in the rhabdomere and a positive component resulting from neurotransmitter-dependent hyperpolarization of second-order neurons in the lamina that are postsynaptic to the photoreceptor cells. The synaptic response of laminal neurons results in transient upward and downward deflections in the ERG trace upon the initiation and cessation, respectively, of the light stimulus (commonly referred to as "ON" and "OFF" transients, respectively; Figure 2A). The ON/OFF transients are dependent upon release of the chemical neurotransmitter histamine from the photoreceptor terminal in response to light (BURG *et al.* 1993). Thus, mutants that are capable of light-induced depolarization of the photoreceptor cells but are defective in synaptic transmission will specifically lack ON/OFF transients but will retain the sustained phototransduction component of the ERG. Further, because the *ey-GAL4* line used to drive mitotic recombination in the *EGUF/hid* system is not expressed in the lamina, recessive mutations conferring ON/OFF transient defects recovered from our screen will be specific to the presynaptic photoreceptor neurons.

This analysis led to the identification of 32 mutations that preferentially affect the ON/OFF transients of the ERG. These synaptic transmission defective mutants were given the allele designations *sd*¹⁻³². Many of the mutants recovered in this analysis lack ON/OFF transients under all conditions tested, as exemplified by mutant *sd*³ (Figure 2B; Table 1). However, in contrast to wild-type flies, which maintain robust ON/OFF transients during repetitive light stimulation (Figure 2C), six mutants in our collection exhibit ON and/or OFF transients that are progressively lost during repetitive light stimulation when measured after a period of dark adaptation, as exemplified by mutant *sd*¹⁵ (Figure 2D; Table 1). Finally, four mutants in our collection retain ON transients but exhibit a progressive loss of the OFF transient upon repetitive light stimulus, as shown for the *sd*¹⁰ mutant (Figure 2E; Table 1).

Complementation analysis and mapping of synaptic transmission mutants: The 32 synaptic transmission mutants recovered from this screen were crossed to one another in all possible combinations to identify allelic relationships. Because each of the chromosomes recovered from this screen confers a recessive lethal phenotype that may derive from the mutation responsible for the synaptic transmission defect, crosses were first

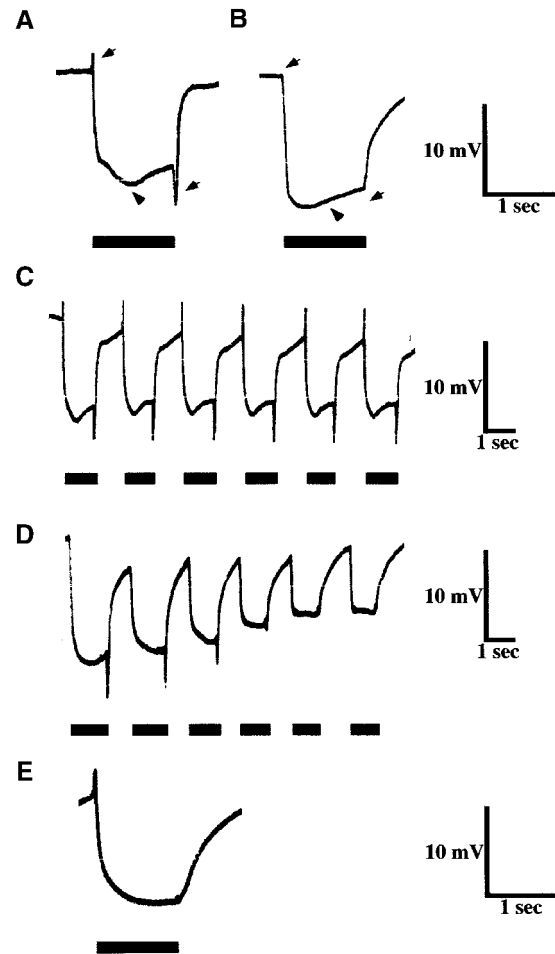


FIGURE 2.—Electrophoretogram recordings of selected mutants recovered in this analysis. All ERG recordings were carried out on animals bearing homozygous eye clones of the relevant chromosome obtained from crosses to the *EGUF/hid* 3R stock. (A) ERG recording from a wild-type control animal bearing the parental chromosome used in genetic analysis showing the ON and OFF transients (arrows) and sustained component (arrowhead) of the ERG. Light pulses are designated by horizontal black bars shown below the ERG recordings. (B) ERG recording from the *sd*³ mutant recovered in this analysis. Note the absence of the ON and OFF transients (arrows) and retention of photoreceptor cell depolarization (arrowhead) in this mutant. (C) ERG recording from a wild-type animal stimulated repetitively with ~1-sec duration each. (D) ERG recording from the *sd*¹⁵ mutant stimulated with intermittent light and dark pulses of ~1-sec duration each. This mutant lacks an ON transient, but retains an OFF transient that is lost gradually upon repetitive stimulation. (E) ERG recording from the *sd*¹⁰ mutant after intermittent light and dark pulses of 0.5 sec each for 10 sec, revealing the absence of only the OFF transient.

carried out to identify mutations that fail to complement each other for a recessive lethal phenotype. However, offspring from those crosses that produced viable *trans*-heterozygotes were also tested for phototactic and ERG phenotypes. Results of this analysis indicate that these 32 mutants represent 14 different complementation

TABLE 1
Electroretinogram phenotypes

Defective ON/OFF transients	Defective ON transient and activity-dependent loss of OFF transient	Activity-dependent loss of OFF transient only
<i>sd</i> ¹ , <i>sd</i> ² , <i>sd</i> ³ , <i>sd</i> ⁴ , <i>sd</i> ⁵ , <i>sd</i> ⁶ , <i>sd</i> ⁷ , <i>sd</i> ⁸ , <i>sd</i> ⁹ , <i>sd</i> ¹¹ , <i>sd</i> ¹² , <i>sd</i> ¹³ , <i>sd</i> ¹⁴ , <i>sd</i> ¹⁶ , <i>sd</i> ¹⁷ , <i>sd</i> ¹⁸ , <i>sd</i> ²⁰ , <i>sd</i> ²¹ , <i>sd</i> ²² , <i>sd</i> ¹⁴ , <i>sd</i> ²⁵ , <i>sd</i> ²⁷ , <i>sd</i> ²⁸ , <i>sd</i> ²⁹ , <i>sd</i> ³⁰ , <i>sd</i> ³¹	<i>sd</i> ¹⁵ , <i>sd</i> ¹⁹	<i>sd</i> ¹⁰ , <i>sd</i> ²³ , <i>sd</i> ²⁶ , <i>sd</i> ³²

groups with between one and six alleles each (Table 2). The large fraction of complementation groups with only a single allele indicates that this screen was not saturating despite the high dose of mutagen used and the large number of mutagenized animals tested. Of the 8 complementation groups with more than one allele each, 6 produce *trans*-heterozygous lethal phenotypes, and 2 appear to be viable as *trans*-heterozygotes. While many of the allelic combinations of complementation group 1 are lethal as *trans*-heterozygotes, several allelic combinations result in viable offspring that are blind and lack ON/OFF transients (Table 2). These viable combinations indicate that at least several of the complementation group 1 mutations represent hypomorphic alleles. Although no definitive conclusion can be drawn from the 8 complementation groups with a single allele each, recombinational and deficiency mapping (see below) suggests that at least 2 of these complementation groups define genes that are essential for viability.

The synaptic transmission mutants were mapped in two ways. All of the mutants were first crossed to a collection of stocks, each bearing a deficiency mapping to the right arm of chromosome 3. This collection of stocks, in aggregate, carries deletions of ~85% of the sequences on 3R. The deficiency stocks were used to localize mutations responsible for recessive lethal phenotypes. Second, all of the mutations were mapped by recombination using a chromosome containing multiple recessive markers. Recombinational mapping was carried out to verify that mutations responsible for recessive lethal phenotypes map close to, and thus likely represent, the synaptic transmission mutations. Synaptic transmission mutations that complemented all deficiencies in the collection or produced conflicting results in the deficiency and recombinational mapping analyses (indicating that the mutation identified from deficiency mapping is incidental) were again crossed to stocks bearing deficiency chromosomes in the regions implicated from recombinational mapping. Offspring from this cross were analyzed for phototactic and ERG phenotypes to investigate the possibility that these mutants are viable or semiviable as hemizygotes. Results of this analysis are summarized in Table 3.

Phenotypic analysis of synaptic transmission mutants: While all of the mutants recovered from our screen

have presynaptic defects in synaptic transmission, this phenotype could arise either from defective neurotransmitter release from the photoreceptor neurons or from failure of the photoreceptor neurons to properly form synaptic connections with their targets in the lamina. To distinguish between these possibilities, the photoreceptor axonal projection patterns of the synaptic transmission mutants were analyzed in head sections using the photoreceptor-specific monoclonal antibody mAb24-B10. Results of this analysis using a parental control line are shown in Figure 3A. Identical experiments carried out with mutations representing the 14 complementation groups recovered from our screen revealed 3 complementation groups that exhibit a grossly abnormal

TABLE 2
Complementation analysis

Complementation groups	Mutations	Viable as <i>trans</i> -heterozygote?
1	<i>sd</i> ⁸ , <i>sd</i> ¹³ , <i>sd</i> ¹⁴ , <i>sd</i> ¹⁷ , <i>sd</i> ²⁴ , <i>sd</i> ³¹	No ^a
2	<i>sd</i> ¹² , <i>sd</i> ¹⁶ , <i>sd</i> ²⁰ , <i>sd</i> ²⁹	No
3	<i>sd</i> ¹⁰ , <i>sd</i> ²³ , <i>sd</i> ²⁶ , <i>sd</i> ³²	No
4	<i>sd</i> ¹ , <i>sd</i> ¹¹ , <i>sd</i> ²¹	Yes
5	<i>sd</i> ¹ , <i>sd</i> ⁶ , <i>sd</i> ¹⁸	No
6	<i>sd</i> ² , <i>sd</i> ¹⁵	Yes
7	<i>sd</i> ²² , <i>sd</i> ²⁷	No
8	<i>sd</i> ⁷ , <i>sd</i> ²⁵	No
9	<i>sd</i> ²⁸	NA
10	<i>sd</i> ³⁰	NA ^b
11	<i>sd</i> ¹⁹	NA
12	<i>sd</i> ³	NA ^{b,c}
13	<i>sd</i> ⁵	NA ^c
14	<i>sd</i> ⁹	NA

NA, not applicable.

^a Three allele combinations in this complementation group are lethal as *trans*-heterozygotes, specifically, *sd*⁸/*sd*¹⁷, *sd*¹⁴/*sd*¹⁷, and *sd*¹⁷/*sd*²⁴. All other combinations produce viable *trans*-heterozygotes that are blind and exhibit ON/OFF transient defects, except mutant *sd*³¹, which exhibits an ON/OFF transient phenotype only *in trans* to *sd*¹⁷.

^b Rare homozygous adult escapers that lack ON and OFF transients are observed.

^c These mutations are lethal in combination with deficiencies that map to the same region.

TABLE 3
Meiotic and deficiency mapping results

Complementation group	Recombinational map position (corresponding polytene localization)	Noncomplementing deficiencies ^a (deletion breakpoints)	Overlapping complementing deficiencies ^b (deletion breakpoints)	Map position ^c
1	86.5 ± 7.4 (94–98)	<i>Df(3R)XS^{1,c}</i> (096A01-07;096A21-25), <i>Df(3R)orb87-5^{1,c}</i> (095F07;096A17-18)	<i>Df(3R)96B</i> (096A21;096B08-10)	96A01-96A18
2	82.4 ± 3.8 (94-95)	<i>Df(3R)3450^d</i> (098E03;099A06-08)	<i>Df(3R)Dr-ru1</i> (099A01-02;099B06-11)	98E03-99A02
3	81 ± 0.4 (95–95)	<i>Df(3R)mbc-R1^d</i> (095A05-07;095D06-11)	<i>Df(3R)mbc-30</i> (095A05-07;095C10-11)	95C10-95D11
4	53.6 ± 13.9 (69–92)	<i>Df(3R)M-KxI^e</i> (086C01;087B01-05), <i>Df(3R)M86D^f</i> (86D01-02;86D04)	<i>Df(3R)T-32</i> (086E02-04;087C06-07)	86D01-86D04
5	63 ± 0.1 (91)	<i>Df(3R)DG^g</i> (090D02-04;090F03-06), <i>Df(3R)PI4^h</i> (090C02-D01;091A01-02)	<i>Df(3R)Cha7</i> (90F01-02;91E5)	90D02-90F02
6	47.6 ± 0.6 (77–85)	None		82B/85
7	54.2 ± 3.5 (86–89)	<i>Df(3R)M-KxI^d</i> (086C01;087B01-05), <i>Df(3R)T-32^d</i> (086E02-04;087C06-07)		86E02-87B05
8	49 ± 3.1 (76–87)	None		82B/87
9	52.8 ± 3.5 (85–88)	None		85-88
10	49.4 ± 0.785 (85–86)	<i>Df(3R)by62^d</i> (085D11-14;085F06), <i>Df(3R)by10^d</i> (085D08-12;085E07-F01)		85D11-85F01
11	101.8 ± 3.4 (99–100)	None		99-100
12	45.6 ± 10.4 (68–88)	<i>Df(3R)dsx2M^d</i> (084C01-03;084E01), <i>Df(3R)Antp17^d</i> (084B01-02;084D11-12 or A06,D14), <i>Df(3R)Scx4^d</i> (084B03;084D01-02), <i>Df(3R)Antp1^d</i> (084B03;084D01-02)	<i>Df(3R)p13</i> (084F02;085B01)	84C03-84D02
13	46.4 ± 2.9 (71–86)	<i>Df(3R)p40^d</i> (084E08-09;085B06), <i>Df(3R)CAI^d</i> (084E12-13;085A06-11), <i>Df(3R)CA3^d</i> (084F02;085A05-07)		84E12-84F02
14	68.3 ± 1.1 (92)	None		92

^aNoncomplementing deficiencies are those that fail to complement a recessive lethal phenotype or a phototactic phenotype and map to the region implicated by recombinational mapping.

^bDeficiencies that complement the synaptic transmission mutations but overlap with noncomplementing deficiencies.

^cPolytene map positions defined by deficiency and/or recombinational mapping (see text). Polytene map positions inferred solely from recombinational mapping are underlined.

^dDeficiency that fails to complement recessive lethal phenotype.

^eDeficiency that fails to complement phototactic and ERG phenotypes.

^fLeft limit determined by *FRT* location at polytene position 82B.

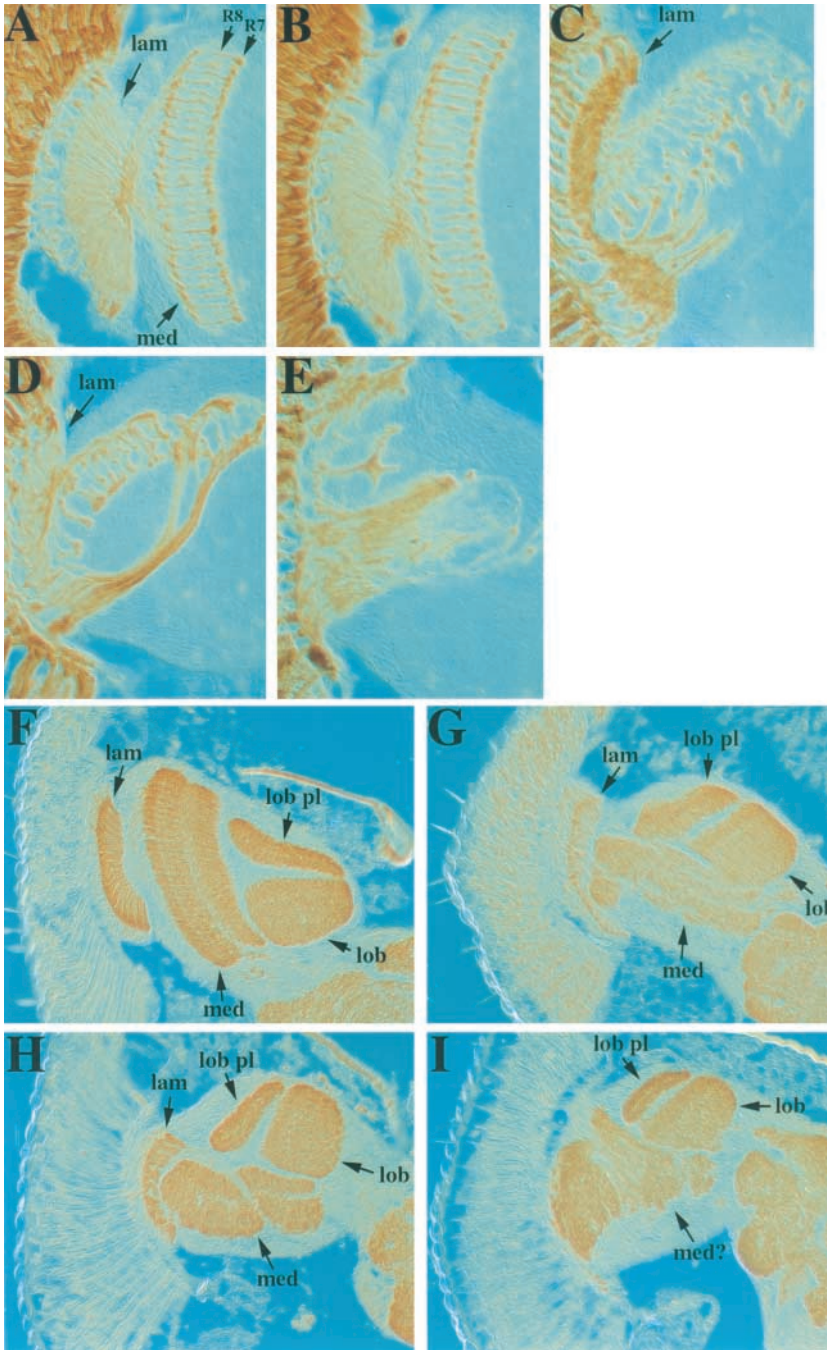


FIGURE 3.—Photoreceptor axon and optic lobe neuropil labeling of homozygous eyes of synaptic transmission mutants. (A–I) Cryostat sections of *Drosophila* heads homozygous in the eye for the indicated genotypes labeled with the photoreceptor axon marker mAb24-B10 (A–E) or the synaptic vesicle-specific antibody synaptotagmin that exclusively labels neuropil (F–I). (A) Parental control showing the ordered array of R1-6 photoreceptor axon labeling in the lamina and R7-8 labeling in the medulla. (B) Complementation group 1 (*sd³¹*) exhibiting an axon projection pattern essentially indistinguishable from control. (C) Complementation group 2 (*sd¹⁶*), (D) complementation group 7 (*sd²²*), and (E) complementation group 9 (*sd²⁸*) all show grossly abnormal photoreceptor projection patterns. (F) Parental control showing the normal optic neuropil pattern. (G) Complementation group 2 (*sd¹⁶*), (H) complementation group 7 (*sd²²*), and (I) complementation group 9 (*sd²⁸*) all show grossly abnormal laminas and medullas. In G and H the medulla is $\sim 90^\circ$ from its normal position relative to the lamina. In I the lobula and lobula plate are $\sim 90^\circ$ from their normal position relative to the rhabdomeres, suggesting that in all three cases some rotational event of the medulla, lobula, and lobula plate has occurred abnormally. lam, lamina; med, medulla; lob, lobula; lob pl, lobula plate.

axonal projection pattern (Figure 3, C–E). Additionally, development of the lamina, which requires innervation by retinal neurons, is partially disrupted in mutant *sd¹⁶* and *sd²²* (corresponding to complementation groups 2 and 7, respectively) and is completely disrupted in mutant *sd²⁸* (corresponding to complementation group 9). Similar axonal and laminal disruption was observed in all other alleles corresponding to complementation groups 2 and 7, confirming the role of these complementation groups in photoreceptor axon pathfinding and/or synaptogenesis. All of the remaining complementation groups exhibit an axonal projection pattern

that is indistinguishable from that of the control line used in this analysis (Figure 3B; data not shown). However, it remains possible that subtle defects in axon pathfinding/synaptogenesis that are below the level of detection of this analysis are present in these mutants.

To further characterize the three complementation groups exhibiting altered photoreceptor axonal pathfinding/synaptogenesis, head sections from representative mutants corresponding to these complementation groups were stained with an antiserum to the synaptic vesicle protein synaptotagmin. This antiserum specifically labels the neuropil in the optic lobe of control

TABLE 4
Lethal-phase analysis of synaptic transmission mutants

Complementation group	Allele	Hemizygous lethal phase ^{a,b}	Homozygous lethal phase ^b
1	<i>sd</i> ⁸	First	Second
	<i>sd</i> ¹³	Viable adults	Pharate adults
	<i>sd</i> ¹⁴	First	Second
	<i>sd</i> ¹⁷	First/second	Second
	<i>sd</i> ²⁴	Viable adults	Viable adults
	<i>sd</i> ³¹	Viable adults	First
2	<i>sd</i> ¹²	Embryo	Embryo
	<i>sd</i> ¹⁶	Embryo/first	Embryo
	<i>sd</i> ²⁰	Embryo	Embryo
	<i>sd</i> ²⁹	Embryo	Embryo
3	<i>sd</i> ¹⁰	Pharate adults	Pharate adults
	<i>sd</i> ²³	Pupae	First
	<i>sd</i> ²⁶	Pupae	First
	<i>sd</i> ³²	Pupae	Third
5	<i>sd</i> ⁴	Second	Second/third
	<i>sd</i> ⁶	Second	Second
	<i>sd</i> ¹⁸	Second	Third instar
7	<i>sd</i> ²²	Embryo/first	Embryo/first
	<i>sd</i> ²⁷	Embryo	Embryo
8	<i>sd</i> ⁷	NA	First
	<i>sd</i> ²⁵	NA	Embryo
9	<i>sd</i> ²⁸	NA	Second-third
10	<i>sd</i> ³⁰	Pupae	Pupae/adult (rare escapers)
11	<i>sd</i> ¹⁹	NA	First-second
12	<i>sd</i> ³	First	Pupae/adult (rare escapers)
13	<i>sd</i> ⁵	Third/pupae	Third/pupae
14	<i>sd</i> ⁹	NA	First

NA, not applicable.

^a Lethal phase *in trans* to a noncomplementing deficiency.

^b First, second, and third indicate first, second, and third instar larval lethal phases, respectively.

flies (Figure 3F). In contrast to the staining observed in control flies, the representatives of complementation groups 2, 7, and 9 showed an altered synaptotagmin labeling pattern (Figure 3, G–I). Only partial laminal staining was observed in the mutants corresponding to complementation groups 2 and 7, and the normal ordered morphology of the medulla is disrupted. Furthermore, while the location of the lamina relative to the overlying photoreceptors is normal in these two complementation groups, the remainder of the optic lobe is misoriented ~90 degrees relative to the lamina. The morphology of the lamina/medulla region of complementation group 9 is even more irregular than that in complementation groups 2 and 7 and exhibits a similar disruption of the orientation of the medulla with respect to the lamina.

The lethal phase of synaptic transmission mutants was determined by placing mutations *in trans* to a noncomplementing deficiency and monitoring the stage at which lethality occurred. For those mutants that do not map to a deficiency, lethal-phase analysis was conducted by monitoring homozygotes. A risk associated with lethal-phase analysis of homozygotes is that the lethal

phase may correspond to or be influenced by incidental mutations residing on the same chromosome. Such an occurrence is likely indicated for mutations conferring a more severe phenotype as a homozygote than as a hemizygote (as seen for mutations *sd*¹³, *sd*²³, *sd*²⁶, *sd*³¹, and *sd*³²). Results of this analysis are summarized in Table 4.

DISCUSSION

We have conducted a screen using the EGUF-hid system to identify recessive mutations that disrupt synaptic transmission in the *Drosophila* visual system. This system generates flies bearing eyes composed exclusively of homozygous clones for a selected chromosome arm and provides an efficient means for conducting F₁ screens for mutations affecting neuronal structure and function, even if these mutations affect genes that are essential for adult viability. In this study we screened 42,500 mutagenized flies and recovered 32 mutants representing 14 complementation groups that preferentially affect the ON/OFF transient component of the ERG. Most of the mutations recovered also confer a recessive lethal phenotype, suggesting that these genes may play

a general role in synaptic transmission in the nervous system. However, at least 4 complementation groups (4, 6, 10, and 12) produce viable adults in *trans*-heterozygous or hemizygous configuration (Table 2). These mutations may reside in genes that function only in the visual system or may affect genes that function more broadly but only detectably affect the visual system. For the remaining complementation groups, it is not possible at this time to conclude definitively whether the genes are essential for viability.

The largest category of mutations in our collection confers ON/OFF transient defects in the visual system without detectably altering the normal pattern of photoreceptor axonal projection to targets in the lamina and medulla. Because the *EGUF-hid* system produces homozygous clones only in the photoreceptor cells, whereas cells in the optic lobe remain heterozygous, the recessive phenotypes induced by this collection of mutations must derive from presynaptic defects in synaptic transmission. The fact that these mutants exhibit a substantial sustained component of the ERG indicates that the molecular defect in these mutants derives from a failure in the release of the chemical neurotransmitter histamine from the photoreceptor nerve terminal at a step downstream of light-induced depolarization. Previous analyses have shown that mutants with defects in histamine biosynthesis and synaptic vesicle fusion and recycling behave identically to this category of mutant (BURG *et al.* 1993; LITTLETON *et al.* 1998; STOWERS and SCHWARZ 1999; SANYAL *et al.* 2001). Thus, genes involved in the reuptake and packaging of histamine into synaptic vesicles or the targeting, fusion, or recycling of synaptic vesicles at release sites are likely represented among these mutants.

A number of our mutations that show normal axonal morphology map to regions encompassing *Drosophila* genes implicated in synaptic vesicle trafficking and thus these genes represent candidate genes to those mutations recovered in our screen (Figure 4; black text). For example, the *sd³* mutant maps to a deficiency that removes the genes encoding the synaptic vesicle-recycling component LAP and the calcium-binding protein synaptotagmin IV. Likewise, complementation group 3 mutants are lethal in combination with deficiencies that remove the genes encoding the synaptic vesicle recycling adaptor protein AP1 σ , the trafficking proteins RAB7 and SEC10, and the t-SNAREs syntaxin1A and syntaxin 18. Complementation analysis revealed two different alleles of the *lap* gene, *lap^{KG06751}* and *lap¹*, which failed to complement and weakly complemented the recessive lethal phenotype of *sd³*, respectively, indicating that *sd³* is a new allele of *lap*. The weak complementation of *lap¹* with *sd³* and occasional appearance of homozygous *sd³* and *lap¹* adult escapers likely reflects the fact that these mutations are hypomorphic alleles of *lap*. By contrast, the lack of homozygous viability and severe

molecular nature of the *lap^{KG06751}* allele (which bears a transposon insertion early in the coding sequence of the first exon of *lap*) indicates that this allele may represent a *lap* null. Further complementation testing excluded the *syntaxin1A* gene as a candidate of complementation group 3. However, *AP1 σ* , *rab7*, *syntaxin18*, and *sec10* mutants have not previously been identified, and thus further work will be required to test whether mutations in these genes underlie the phenotypes we have documented. While most of the candidate genes listed in Figure 4 correspond to axonal pathfinding components and components of the vesicle trafficking pathway, the recent identification of *milton*, a *Drosophila* gene involved in the transport of mitochondria to synaptic terminals, from an *EGUF/hid* screen demonstrates that our mutants could represent a much broader collection of genes than those displayed in Figure 4 (STOWERS *et al.* 2002).

A subset of the mutants recovered in this analysis retains some residual synaptic function that is lost upon repetitive stimulation. Similar phenotypes have been shown to result from mutations in genes that function in synaptic vesicle priming or recycling, including *Shibire*, *NSF1*, and *Endophilin1A* (SALKOFF and KELLY 1978; POODRY and EDGAR 1979; KOENIG and IKEDA 1989; KAWASAKI *et al.* 1998; RIKHY *et al.* 2002). This phenotype is thought to arise from a reduced ability to maintain a readily releasable pool of synaptic vesicles, which is revealed only upon rapid repetitive stimulation. Thus, the complementation groups displaying these phenotypes may encode products that participate in synaptic vesicle priming or recycling. Although it remains unclear why several mutants in this category exhibit activity-dependent loss of only the OFF transient, it is worth noting that a similar phenotype was reported for the synaptic vesicle recycling mutant, *endophilin1A*, demonstrating that this characteristic is not incompatible with a role in synaptic vesicle trafficking.

The other major category of mutant recovered in our screen consists of those that lack ON/OFF transients because the photoreceptor neurons fail to properly project axons to their target cells in the optic lobe. The photoreceptor cells in this collection of mutants (complementation groups 2, 7, and 9) fail to choose a single side of the medulla to project down and subsequently extend down both sides. A similar phenotype has been observed for the *irreC-rst* gene (SCHNEIDER *et al.* 1995). The proper extension of the R8 axons down one side of the medulla is needed for the reorientation of the medullar neurons relative to the lamina (WOLFF *et al.* 1997; CLANDININ and ZIPURSKY 2002). Thus, the orientation of the lamina and medulla that is established in third instar larvae is incorrectly retained in the adult (compare Figure 3F to Figure 3, G–I). For complementation groups 2 and 7, the photoreceptor R8 axons appear to have extended properly through the laminal precursor cells, as this extension is believed necessary

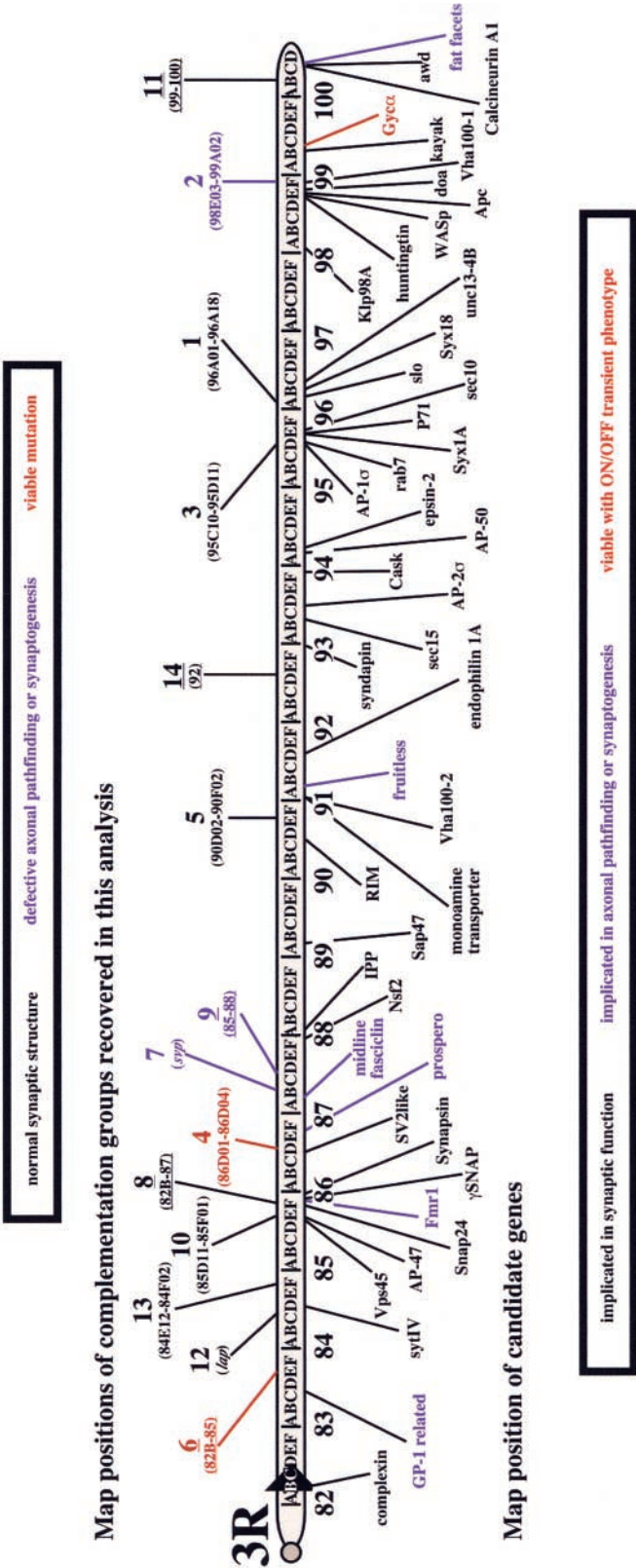


FIGURE 4.—Map positions of synaptic transmission mutants identified in this analysis and candidate genes residing on the same chromosome arm. Synaptic transmission mutants recovered in this study are depicted above the chromosome arm and candidate genes are shown below. The FRT element used in this analysis is depicted as a black triangle near the centromere. The map positions (in polytene units) of the complementation groups recovered in this work are designated. Those complementation groups that have been localized by recombination only are underlined. Complementation groups that do not affect synaptic structure are designated in black. Complementation groups designated in purple represent those conferring axonal pathfinding phenotypes. Complementation groups of the neurotransmitter release apparatus are viable as *trans*-heterozygotes or hemizygotes. Candidate genes encoding components of the neurotransmitter release apparatus are designated in red. Candidate genes involved in axonal pathfinding are designated in purple. Candidate genes that are not essential for viability are shown in red.

to induce cell division in the lamina. In contrast, complementation group 9 mutants appear to lack laminal neurons, indicating that R8 axon extension failed prior to reaching the lamina.

Several of the mutants that show axon projection defects also map to regions containing candidate genes implicated in axonal pathfinding (Figure 4; those highlighted in purple). For example, complementation group 2 alleles map to a deficiency that removes the *WASp*, *Doa*, *Apc*, and possibly the *Huntingtin* genes. These genes have been implicated in sensory organ development and neuronal maintenance (YUN *et al.* 1994; AHMED *et al.* 1998; DRAGATIS *et al.* 2000; BEN-YAACOV *et al.* 2001). Complementation groups 7 and 9 map to a region that encompasses the *prospero* and *svp* genes, which are known to be involved in axonogenesis and neurogenesis (MLODZIK *et al.* 1990; VAESSIN *et al.* 1991; HU *et al.* 1998). Localization of complementation group 9 is based solely on meiotic mapping criteria, as deficiencies removing the *sd²⁸* allele were not identified. Thus, complementation group 9 spans a large region of chromosome 3R and many additional candidate genes exist for this complementation group. Complementation analysis with *prospero* mutations excluded this gene as a candidate of complementation groups 7 and 9. However, both alleles of complementation group 7 fail to complement the recessive lethal phenotype of two different *svp* mutations (*svp¹* and *svp⁰⁷⁸⁴²*), indicating that complementation group 7 represents the *svp* gene. *svp* encodes a steroid receptor involved in photoreceptor cell fate specification. Loss of *svp* function results in failure to form R3/4 and R1/6 photoreceptors with the corresponding cells defaulting to an R7 fate (MLODZIK *et al.* 1990). The absence of R3/4 and R1/6 photoreceptors or the overabundance of R7 photoreceptors may be responsible for the abnormal axonal projections seen in these mutants. While further complementation analysis has excluded the *WASp*, *Doa*, and *Apc* genes as complementation group 2 candidates, the *Huntingtin* gene remains a candidate that will be investigated in future work.

While many of the mutants recovered in this analysis map to genomic regions bearing candidate genes, a number of genes on chromosome 3R that are known from previous work to participate in photoreceptor development and function were not recovered in this analysis. For example, we did not recover mutations in *endophilin1A*, *syntaxin1A*, or *gyca* despite the fact that previous work has shown that mutations in these genes can result in ERG phenotypes like those obtained in our screen (LITTLETON *et al.* 1998; GIBBS *et al.* 2001; RIKHY *et al.* 2002). Given the frequency of obtaining recessive lethal mutations at the dosage of mutagen used in our analysis (GRIGLIATTI 1998) and current estimates indicating that approximately one-third of the ~3000 genes residing on the right arm of chromosome 3 can be mutated to produce a recessive lethal pheno-

type (MIKLOS and RUBIN 1996), we conservatively estimate that an average of at least 10 alleles per essential gene should have been recovered. However, the largest complementation group in our collection consists of only six alleles, and 6 of the 15 complementation groups recovered are represented by only one allele. These findings indicate that our screen did not reach saturation, despite the large number of mutagenized animals analyzed.

The lack of saturation in our screen may derive from several different sources. Because phototactic selection was conducted with a population of flies, the majority of which were probably not blind, many blind mutants may have been swept toward the light source as part of the "herd." Thus, many mutants may have been lost because they behaved like flies with normal vision. Alternatively, the lack of saturation observed may derive from a selection bias favoring the recovery of only particular alleles of genes that function in photoreceptor development and function. For example, null mutations of the *syntaxin1A* gene fail to support retinal development (STOWERS and SCHWARZ 1999), and thus such alleles would not have been recovered from our screen given our requirement that mutants exhibit normal external eye morphology. By contrast, hypomorphic alleles of genes required for cell viability may not reduce synaptic function sufficiently to allow their recovery from a phototaxis screen. Thus, only a restricted subset of hypomorphic alleles of such genes would be expected from our screen. While selection bias cannot be circumvented, conducting the EGUF/hid screen under conditions favoring the recovery of conditional alleles would likely extend the range of genes that could be obtained. Temperature-sensitive alleles of the *shibire* and *NSF1* genes result in blindness and a lack of ON/OFF transient phenotypes at restrictive temperature when they are made homozygous in the retina using the *EGUF-hid* system (our unpublished results), supporting the validity of such an approach.

In summary, we have identified a collection of mutants that display presynaptic defects in synaptic transmission in the *Drosophila* visual system and have placed the mutants into two broad categories: those with defective photoreceptor axonal projection patterns and those with apparently normal photoreceptor structure. For those mutants that exhibit normal axonal structure, further structure/function studies using more powerful electrophysiological and ultrastructural approaches will be used to better define their roles in synaptic transmission. Additional mapping to further narrow the regions containing these genes, coupled with candidate gene complementation testing, sequencing, and transgenic rescue experiments, will facilitate the molecular identification of genes responsible for these phenotypes. We anticipate that identification of these genes will contribute important insights into the molecular mechanisms of synaptic development and function.

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